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Endophytic association between *Alternaria oxytropis* and *Oxytropis kansuensis* affected by nutritional conditions and temperature

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Abstract

Alternaria oxytropis has been reported as an endophyte of species in the genera *Astragalus* and *Oxytropis* (Fabaceae) in the USA and China. Infected plants contain the alkaloid swainsonine produced by the fungus, and this causes poisoning of grazing animals. Therefore, ecological investigations are important to clarify the extent of this endophytic association. Seeds of *O. kansuensis* were collected from a locality known for indigenous *A. oxytropis* in China and were cultured on artificial media under controlled conditions for about two months. Less than 50% of seeds germinated with colonies of *A. oxytropis*, however, germination and seedling growth were influenced by the type of culture media used as well as temperature. Seedling growth with colonies of the fungus was significantly better on low nutrient media at low temperature (20 °C) than that on a high nutrient media at high temperature (25 °C). Seedlings with colonies of the fungus on a high nutrient media were eventually killed by the fungus. It is suggested that the endophytic association between the fungus and plants is affected by nutritional conditions and temperature.

Key words – Alkaloid – Endophyte – Host-parasite interaction – Poisonous plant – Seed germination – Swainsonine

Introduction

A new endophytic fungus originally isolated from *Oxytropis kansuensis* collected in Qinghai, China, was described as *Embellisia oxytropis* Q. Wang, Nagao & Kakish. (Wang et al. 2006). Pryor et al. (2009) transferred this species to a new genus, *Undifilum* based on phylogenetic analyses and conidium germ-tube morphology. However, Woudenberg et al. (2013) renamed the fungus as *Alternaria oxytropis* (Q. Wang, Nagao & Kakish.) Woudenb. & Crous based on phylogenetic analysis using sequence data of multi-regions of rDNA. This species has been reported to have endophytic associations with eight species of *Astragalus* and three species of *Oxytropis* in the western USA (Braun et al. 2003, Ralphs et al. 2008, 2011), and with two species of *Astragalus* and six species of *Oxytropis* in the highlands of China (Wang et al. 2006, Yu et al. 2010, Zhou et al. 2013). Plants containing this endophyte cause poisoning of grazing animals because the alkaloid

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swainsonine is produced by the fungus (Molyneux & James 1982, James et al. 1989, Stegelmeier et al. 1999, Ralphs et al. 2002, Zhao et al. 2009, Gao et al. 2012). Control measures are thus important for safe grazing.

Alternaria oxytropis has been detected from all parts of plants (roots, leaves, stems, seeds and flowers) (Wang et al. 2006, Cook et al. 2009, Reyna et al. 2012). Oldrup et al. (2010) reported that the fungus was localized in the seed coat of infected plants and demonstrated its transmission to seedlings with seed embryo cultures of *Astragalus lentiginosus* and *O. sericea* collected in Utah, USA. Ralphs et al. (2011) also demonstrated that the fungus was transmitted to seedlings via seeds. They planted seeds of *A. lentiginosus*, *A. mollissimus*, *A. wootonii*, and *O. sericea* collected in the western USA in containers with sand and peat moss and detected swainsonine after harvesting plants, although the detection rate was low. Therefore, the fungus transmits to new plants via seeds and spreads in all tissues of plants. However, the effects of environmental conditions for transmission and infection of the fungus are not clearly understood.

In this study we collected seeds of *O. kansuensis* from the type locality of the fungus (Qinghai, China), and carried out seed germination experiments on artificial media to clarify the effects of nutritional conditions and temperature for transmission of the fungus to seedings.

Materials & Methods

Seed collection

Plants of *O. kansuensis*, including seeds, were collected in September 2013 at Mt. Riyue, Qinghai, China (36°26′33″N, 101°05′48″E, alt. 3820 m), the type locality of *A. oxytropis*, and kept in a refrigerator at about 4 °C until use. Endophytic infections of these plants with *Alternaria oxytropis* were confirmed by isolating the fungus from the leaves or stems.

Seed culture on media

Seeds of *O. kansuensis* were removed from plants and soaked for 15 min in sterile distilled water. They were then surface-sterilized using 70% ethanol for 1min, followed by 1% NaClO for 3–5 min to remove contaminations. The seeds were then rinsed in sterile distilled water (3 changes), dried on sterilized filter paper for 12 h, and transferred to media in glass beakers (100 ml) containing approximately 30 ml of the artificial medium, potato dextrose agar medium (PDA), water agar medium (WA) or cornmeal agar medium (CMA). PDA medium contained 200 g potato, 20 g glucose, 6 g agar and 1 L deionized water. WA medium consisted of 6 g agar and 1 L deionized water, while CMA medium included 10g cornmeal powder and 1 L deionized water. Three seeds were placed on the media in each beaker, and five of them were used for each medium. They were incubated in a plant growth chamber (MGC-450H) at 20 °C or 25 °C with 12 h light/12 h dark and remained under these conditions for about two months.

Confirmation of *A. oxytropis* in seeds or seedlings

Alternaria oxytropis produces a characteristic dark and slow-growing colony on media, as reported by Wang et al. (2006) and is clearly distinguished from colonies of other contaminating fungi. Therefore, the presence of the fungus was confirmed by observations of colonies growing on media from seeds and seedlings. For confirmation of the species, microscopic observations were also conducted using preparations of mycelia obtained from some colonies.

Results

Germination of seeds

Less than half of the seeds germinated on media under controlled conditions (Table 1), while about 60% of seeds did not germinate or were contaminated with microorganisms except *A. oxytropis* (bacteria or fungi). Contamination rate was higher at 25 °C (WA: 40.0%, CMA: 20.0%, PDA: 40.0%) than at 20 °C (WA: 6.7%, CMA: 3.3%, PDA: 30.0%). Seeds on PDA were also more

highly contaminated (20 °C: 30.0%, 25 °C: 40.0%) than those on WA (20 °C: 6.7%, 25 °C: 40.0%) and CMA (20 °C: 3.3%, 25 °C: 20.0%). Ratio of no germination seed without contamination was higher at 20 °C (WA: 56.7%, CMA: 56.7%, PDA: 33.3%) than at 25 °C (WA: 26.7%, CMA: 13.3%, PDA: 20.0%) on each medium. Germination of seeds with *A. oxytropis* (26.7–40.0%) was mostly higher than that of seeds exclusively germinated (0.0–26.7%) on three media at both temperatures, except on WA at 25 °C (13.3%).

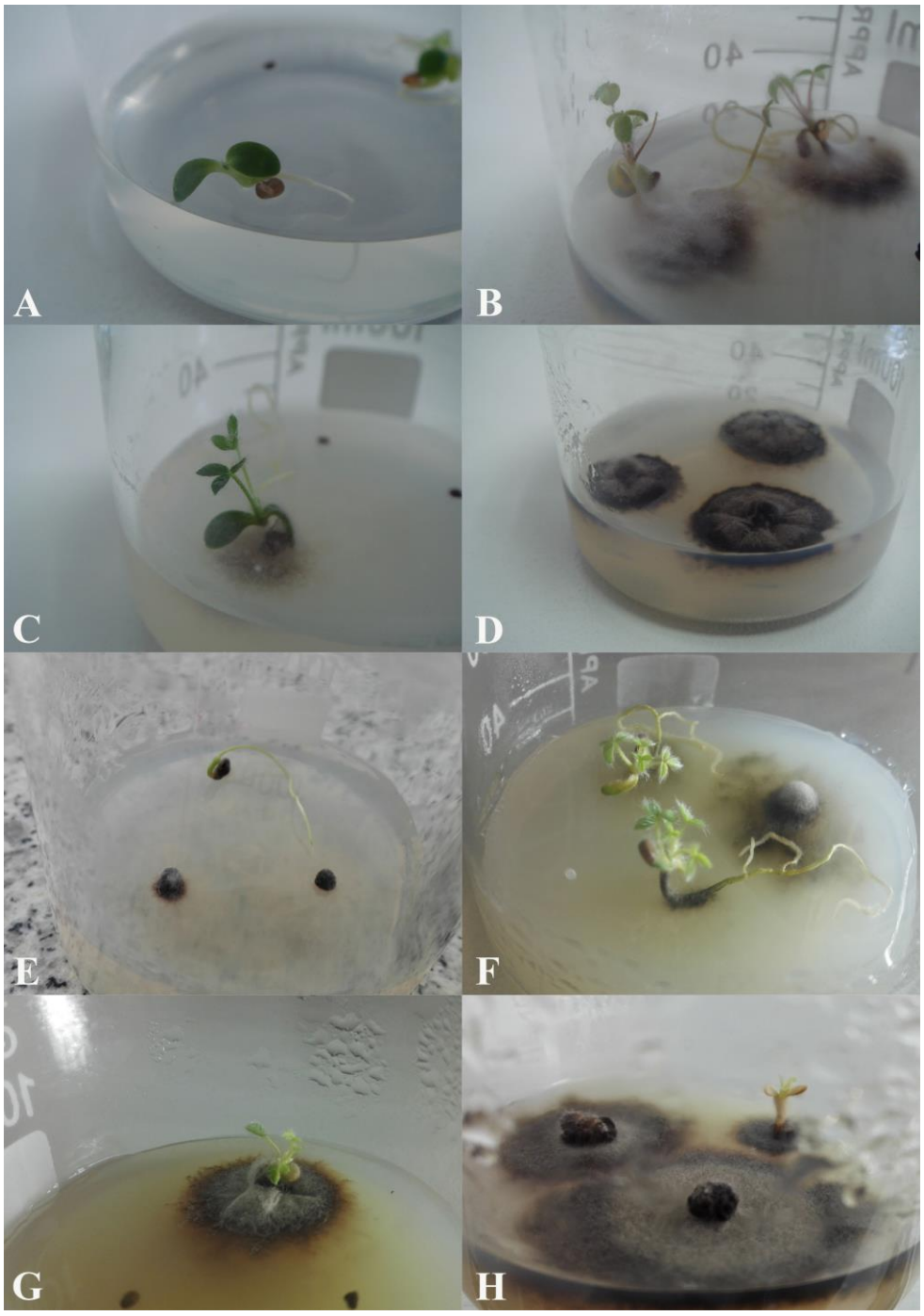

Fig. 1 – **A** Two germinated seeds of *Oxytropis kansuensis* produced seedlings on WA at 20 °C. **B** Growth of two seedlings with *Alternaria oxytropis* on WA at 20 °C. **C** Growth of one seedling with *A. oxytropis* on CMA at 20 °C. **D** Three killed seedlings covered by *A. oxytropis* on PDA at 20 °C. **E** Growth of one seedling with *A. oxytropis* on WA at 25 °C. Two killed seedlings covered by *A. oxytropis*. **F** Growth of two seedlings with *A. oxytropis* on CMA at 25 °C. One killed seedling covered by *A. oxytropis*. **G** Growth of one seedling with *A. oxytropis* on PDA at 25 °C. **H** Three killed seedlings covered by *A. oxytropis* on PDA at 25 °C.

Growth of seedlings

Growth conditions of seedlings after germination with *A. oxytropis* varied between different media and temperatures (Table 2, Fig. 1). Growth conditions were relatively better at 20 °C than 25 °C, and also better on WA or CM than on PDA. The number of seedlings growing with *A. oxytropis* after two months was higher on WA (8 seedlings normally growing/10 total number of seedlings germinated with the fungus) than CMA (5/15) and PDA (1/15), and also higher at 20 ºC (10/28) than 25 °C (4/12). Death of seedlings after two months was higher on PDA (14 dead seedlings /total 15 seedlings) than on WA (2/10) or CMA (10/15), and also higher at 25 ºC (8/12) than 20 ºC (18/28). Colonies of the fungus associated with seedlings on PDA grew fast and reduced the growth of seedlings (Fig. 1D, G, H). Some of these seedlings were eventually killed by the fungus (Fig. 1D, H).

Table 1 Seed condition of *Oxytropis kansuensis* on media (WA, CMA, PDA)* under 20 °C and 25 °C (%)**

Seed condition	WA		CMA		PDA	
	20 °C	25 °C	20 °C	25 °C	20 °C	25 °C
Germination with *Alternaria oxytropis*	26.7	13.3	30.0	40.0	36.7	26.7
Exclusive germination	10.0	20.0	10.0	26.7	0.0	13.3
No germination	56.7	26.7	56.7	13.3	33.3	20.0
Contamination with microorganisms except *A. oxytropis*	6.7	40.0	3.3	20.0	30.0	40.0

*WA: water agar medium, CMA: corn meal agar medium, PDA: potato dextrose agar medium. **Ratio (%) is calculated based on total seed number on each medium and each temperature: 30 and 15 seeds were tested in each medium at 20 °C and 25 °C, respectively.

Table 2 Growth conditions of seedlings* germinated with *Alternaria oxytropis* after 2 months incubation on media (WA, CMA, PDA)** at 20 °C or 25 °C

Seedling	WA		CMA		PDA	
	20 °C	25 °C	20 °C	25 °C	20 °C	25 °C
Total number	8	2	9	6	11	4
Normal growth	6	2	4	1	0	1
Dead seedling	2	0	5	5	11	3

* Number of seedlings is showed among total number of seedlings germinated with *A. oxytropis* in each medium and temperatures. ** WA: water agar medium, CMA: corn meal agar medium, PDA: potato dextrose agar medium.

Discussion

In seed cultures prepared using seeds obtained from *O. kansuensis* and with *A. oxytropis* as an endophyte, some seeds germinated with the fungus on the media but the ratio of seed germination with *A. oxytropis* was variable depending on the media and temperatures (13–40%), and relatively low compared to seeds germinated alone (0–27%), or without germinated seeds (13–57%) and seeds contaminated with microorganisms except *A. oxytropis* (3–40%). Oldrup et al. (2010) and Ralphs et al. (2011) also reported transmission of the fungus via seeds but the rate of transmission was relatively low. Therefore, it is suspected that the fungus does not infect all seeds of the plant and some seeds are free from the fungus.

Associations with the fungus and the plant differ among different culture conditions. With a rich nutrient medium (PDA), the fungus grew very fast around germinating seeds and finally killed the seedling, however, with WA and CMA media, fungal growth was restricted and seedling growth was better than for those on PDA. Therefore, it is suspected that the fungus-host association is affected by nutrient conditions, especially fungal growth conditions. If culture conditions are suitable for fungal vegetative growth, then the fungus does not have an endophytic association with the plant for its survival but rather kills the plants as a pathogen. Temperature also affected their

association. Seedling growth with the fungus at 20 ºC was better than growth at 25 ºC. Although additional experiments below 20 ºC and above 25 ºC are required, temperature is suspected to be an important factor for the plant/endophyte association. Plants of *O. kansuensis* having *A. oxytropis* as an endophyte were collected in Qinghai grassland at an altitude of about 3800 m above sea level. Environmental conditions of this area are very severe for the survival and growth of this plant with low temperature and poor soil nutrition conditions. Therefore, we suspect that the fungus has endophytic associations with the plant under these environmental conditions and that these environmental factors are very important in their association. If conditions change, the endophytic association is highly affected, and the fungus changes its characters to a pathogen. In this experiment, we demonstrated the importance of nutrients and temperature for the endophytic association.

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References

Braun K, Romero J, Liddell C, Creamer R. 2003 – Production of swainsonine by fungal endophytes of locoweed. Mycological Research 107, 980–988. Doi 10.1017/S095375620300813X

Cook D, Gardner DR, Ralphs MH, Pfister JA et al. 2009 – Swainsoninine concentrations and endophyte amounts of *Undifilum oxytropis* in different plant parts of *Oxytropis sericea*. Journal of Chemical Ecology 35, 1272–1278. Doi 10.1007/s10886-009-9710-9

Gao XL, Cook D, Ralphs MH, Yan L et al. 2012 – Detection of swainsonine and isolation of the endophyte *Undifilum* from the major locoweeds in Inner Mongolia. Biochemical Systematics and Ecology 45, 79–85. Doi 10.1016/j.bse.2012.07.012

James LF, Elbein AD, Molyneux RJ, Warren CD. 1989 – Swainsonine and related glycosidase inhibitors. Iowa State University Press, Ames.

Molyneux RJ, James LF. 1982 – Loco intoxication: indolizidine alkaloids of spotted locoweed (*Astragalus lentiginosus*). Science 216, 190–191. Doi 10.1126/science.6801763

Oldrup E, McClain-Romero J, Padilla A, Moya A et al. 2010 – Localization of endophytic *Undifilum* fungi in locoweed seed and influence of environmental parameters on a locoweed in vitro culture system. Botany 88, 512–521. Doi 10.1139/B08-130

Ralphs MH, Creamer R, Baucom D. 2008 – Relationship between the endophyte *Alternaria* spp. and the toxic alkaloid swainsonine in major locoweed species (*Astragalus* and *Oxytropis*). Journal of Chemical Ecology 34, 32–38. Doi 10.1007/s10886-007-9399-6

Ralphs MH, Cook D, Gardner DR, Grum DS. 2011 – Transmission of the locoweed endophyte to the next generation of plants. Fungal Ecology 4, 251–255. Doi 10.1016/j.funeco.2011.03.001

Ralphs M, Welsh S, Gardner D. 2002 – Distribution of locoweed toxin swainsonine in populations of *Oxytropis lambertii*. Journal of Chemical Ecology 28, 701–707. Doi 10.1023/A:1015228507950

Reyna R, Cooke P, Grum D, Cook D, Creamer R. 2012 – Detection and localization of the endophyte *Undifilum oxytropis* in locoweed tissues. Botany 90, 1229–1236. Doi 10.1139/b2012-092

Stegelmeier BL, James LF, Panter KE, Gardner DR et al. 1999 – Dose response of sheep poisoned with locoweed (*Oxytropis sericea*). Journal of Veterinary Diagnostic Investigation 11, 448–456. Doi 10.1177/104063879901100510.

Wang Q, Nagao H, Li Y, Wang H, Kakishima M. 2006 – *Embellisia oxytropis*, a new species isolated from *Oxytropis kansuensis* in China. Mycotaxon 95, 255–260.

Woudenberg JHC, Groenewald JZ, Binder M, Crous PW. 2013 – *Alternaria* redefined. Studies in Mycology 75, 171–212. Doi 10.3114/sim0015

Pryor BM, Creamer R, Shoemaker RA, McClain-Romero J, Hambleton S. 2009 – *Undifilum*, a new

genus for endophytic *Embellisia oxytropis* and parasitic *Helminthosporium bornmuelleri* on legumes. Botany 87, 178–194. Doi 10.1139/B08-130

Yu YT, Zhao Q, Wang J, Wang Y et al. 2010 – Swainsonine-producing fungal endophytes from major locoweed species in China. Toxicon 56, 330–338. Doi 10.1016/j.toxicon.2010.03.020

Zhao BY, Liu ZY, Wang JJ, Sun LS et al. 2009 – Isolation and NMR study on swainsonine from locoweed, *Astragalus strictus*. Agricultural Sciences in China 8, 115–120. Doi 10.1016/S1671-2927(09)60016

Zhou QW, Lu H, He P, Yan DJ et al. 2013 – Distribution of endophytic fungi in *Oxytropis glabra* DC. Acta Microbiologica Sinica 53, 478–485. (in Chinese)